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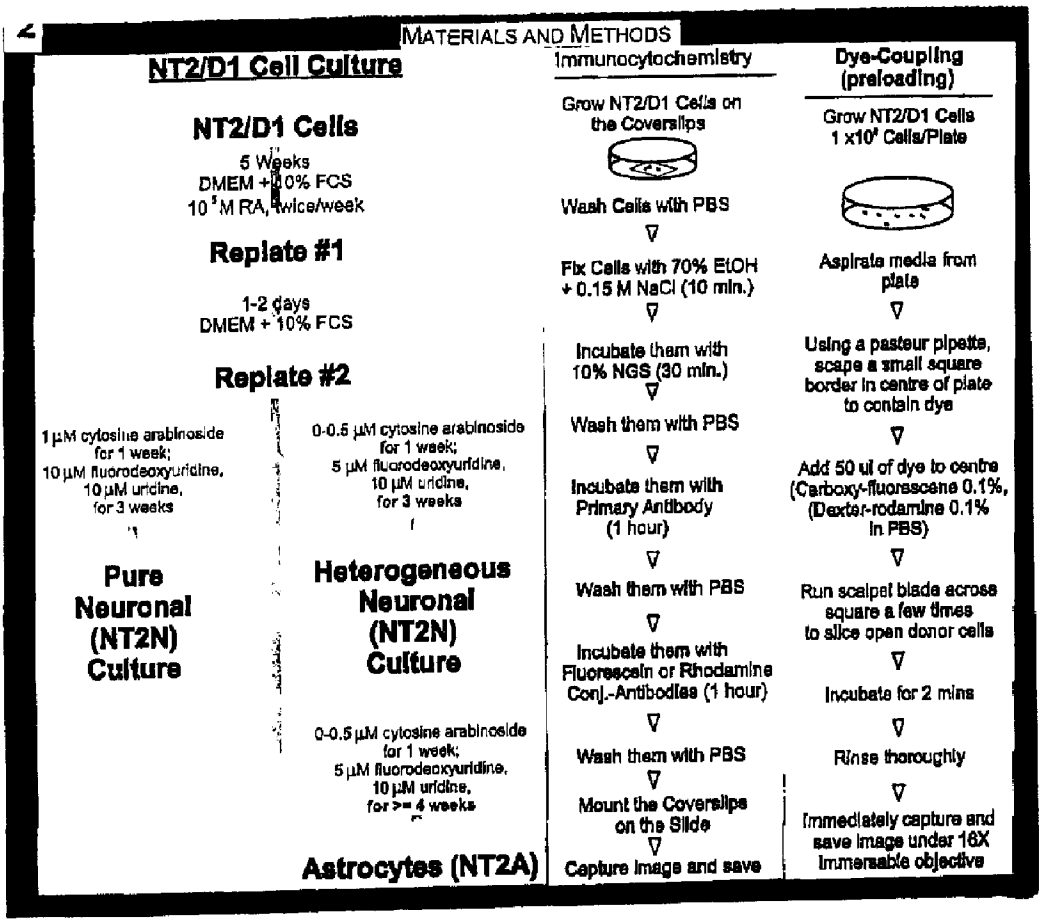

(57) Abrégé/Abstract:
The invention relates to formation of replicative cells from multipotential stem cells. As the most numerous cell type in the brain, astrocytes are coupled via gap junction channels. It is believed that communication among astrocytes is normally regulated by

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(57) Abrégé(suite)/Abstract(continued):
extracellular ions, neurotransmitters and neuromodulators. However, the level of astrocytic coupling is altered in abnormal conditions such as stroke, brain trauma and Alzheimer's disease. A well established human progenitor cell line, NT2/D1, has been previously differentiated into pure neuronal cultures. For the first time, we report the differentiation of NT2/D1 cells into astrocytes, which express connexin43 and are coupled via gap junctions. Thus, human NT2/D1 cells are not merely committed neuronal progenitors but, similar to the embryonal stem cells, they can give rise to both lineages. This has potential in treatment of various forms of neurological deficiency. Other replicative cells may be formed from multipotential stem cells according to the invention.

ABSTRACT

The invention relates to formation of replicative cells from multipotential stem cells. As the most numerous cell type in the brain, astrocytes are coupled via gap junction channels. It is believed that communication among astrocytes is normally regulated by extracellular ions, neurotransmitters and neuromodulators. However, the level of astrocytic coupling is altered in abnormal conditions such as stroke, brain trauma and Alzheimer's disease. A well established human progenitor cell line, NT2/D1, has been previously differentiated into pure neuronal cultures. For the first time, we report the differentiation of NT2/D1 cells into astrocytes, which express connexin43 and are coupled via gap junctions. Thus, human NT2/D1 cells are not merely committed neuronal progenitors but, similar to the embryonal stem cells, they can give rise to both lineages. This has potential in treatment of various forms of neurological deficiency. Other replicative cells may be formed from multipotential stem cells according to the invention.

CELL CULTURE AND METHOD FOR PRODUCTION

FIELD OF THE INVENTION

The present invention relates to a cell culture and to a method for producing replicating cells from multipotential stem cells.

BACKGROUND OF THE INVENTION

As the most numerous cell type in the brain, astrocytes are coupled via gap junction channels. It is believed that communication among astrocytes is normally regulated by extracellular ions, neurotransmitters and neuromodulators. However, the level of astrocytic coupling is altered in abnormal conditions such as stroke, brain trauma and Alzheimer's disease. A well established human progenitor cell line, NT2/D1, has been previously differentiated into pure neuronal cultures.

In U.S. Patents 5,175,103 and 5,654,189 (both attributed to Lee and Pleasure) there are provided a method for producing a stable at least 95% pure population of post-mitotic human neurons expressing exogenous gene products and a culture of such cells that is at least 95% homogeneous produced by culturing NT2 cells with retinoic acid. These patents have, as their goal, an at least 95% pure population of post-mitotic human neurons. There is not an apparent indication that it may be desirable to produce a culture of astrocytes or a mixed culture of astrocytes and neurons.

SUMMARY OF THE INVENTION

Multipotential cells are capable of differentiation to various cell types, some of which may be replicating cells, others which may be non-replicating or post-mitotic. We have found that we can produce replicating cells from cultures of cells which had heretofore been considered to be "committed" to a particular route of differentiation. Thus, according to the invention, multipotential cells are capable of differentiation to various cell types, some of which are replicating cells others are non-replicating.

We have found that we can produce cultures of astrocytes and mixed cultures of astrocytes and neurons from multipotential stem cells. Astrocytes are implicated in the

delivery of nutrients to and the removal of waste from neurons these cultures. Thus, cultures of astrocytes, and astroctyes in combination with neurons are useful in treatment of various forms of neurological deficiency.

The present invention provides a culture of replicating cells produced by (a) culturing multipotential stem cells in a medium containing retinoic acid, (b) incubating the cells from step (a) in a medium containing an anti-mitotic agent at an amount less than that required to kill a replicating cell, and (c) subsequently incubating cells in a medium containing uridine until replicating cells are produced.

Further, the present invention provides a culture of replicating cells produced by (a) culturing multipotential stem cells in a medium containing retinoic acid, (b) incubating the cells from step (a) in a medium containing cytosine arabinoside at an amount less than that required to kill a replicating cell, and (c) subsequently incubating cells in a medium containing uridine until replicating cells are produced.

Conventional methods for forming neurons from multipotential stem cells have not previously allowed formation of replicating cells. Prior art culture methods for obtaining neuronal cells from multipotential stem cells involve incubations with replication-inhibiting substances at levels adequate to kill replicating cells, such as astrocytes and oligodendrocytes, leaving a culture of post-mitotic cells, such a neurons. To obtain a culture of replicating cells, it has surprisingly been found that lower levels of, and even the absence of an anti-mitotic agent, such as cytosine arabinoside, in culture medium results in formation of cultures containing replicating cells, for example, astrocytes and/or oligodendrocytes.

Thus, the invention also provides a process for producing replicating cells from multipotential stem cells comprising the steps of: (a) culturing multipotential stem cells in a medium containing retinoic acid, (b) incubating the cells from step (a) in a medium containing an anti-mitotic agent at an amount less than that required to kill a replicating cell, and (c) subsequently incubating cells in a medium containing uridine until replicating cells are produced.

Additionally, the invention provides a process for producing replicating cells from multipotential stem cells comprising the steps of: (a) culturing multipotential stem cells in a medium containing retinoic acid, (b) incubating the cells from step (a) in a medium containing cytosine arabinoside at an amount less than that required to kill a replicating cell,

and (c) subsequently incubating cells in a medium containing uridine until replicating cells are produced.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the present invention will now be described, by way of example only, with reference to the attached Figures, wherein:

Figure 1 (A to I) shows immunofluorescent localization of MAP2, NF200, Cx43, GFAP and vimentin during differentiation of NT2/D1 cells. The undifferentiated NT2/D1 cells lack MAP2 (A) and NF200 (B) while they express high levels of Cx43 (C). These cells differentiate into a pure MAP2 (D) and NF (E) positive neuronal culture, which lacks Cx43 (F). Alternatively NT2/D1 cells can differentiate into NT2 neurons (red) and GFAP positive astroctyes (Green) (G) when treated with lower concentrations of antiproliferative compounds for > 4 weeks (see text). The mature NT2 astrocytes (red) express Cx43 (green) (H) but they are negative for vimentin (I). Each panel is a separate field of view. Bar - 20 μm (A,B,C,H,I); 50 μm (D,E,F,G).

Figure 2 shows dye coupling in NT2 astrocytes is readily demonstrated, using the scrape loading technique. NT2 astroctyes receive carboxyfluorescein from the brightly fluorescent donor cells labeled with both carboxyfluorescein and dextran tetramethylrhodamine (A). The scrape loaded donor cells have been shown with black arrows (A,B). Some of the less flourescent recipient cells are shown with white arrowheads (A,B). Other cells not receiving the dye at the time the image was captured are shown with asterisk (A,B). Bar = 50 μm.

Figure 3 details Immunofluorescent staining of NT2/D1 cells for Cx43 and MAP2. Before RA induction, the undifferentiated cells express an abundant amount of Cx43 (A) but are negative for MAP2 (B). Bar = 50 μm.

Figure 4 shows that NT2/D1 cells differentiate into a pure (97 ± 2%, n=20) population of MAP2 positive neurons (NT2N) (A) after treatment with RA and antiproliferative agents. These mature neurons lose all Cx43 expression (B). Bar = 20 μm.

Figure 5 shows that NT2/D1 cells can also differentiate into a heterogeneous culture of MAP2 positive neurons (30 ± 3%, n=20), shown in red, and GFAP positive astrocytes, shown in green. Bar = 50 μm.

Figure 6 shows that using a modified method of the invention, the cultures develop into a large number (62 ± 5%, n=20) of mature GFAP positive NT2 astrocytes (NT2A) (A) over a longer period of time. These cultures are negative for vimentin (B). Bar = 750 μm (A), 100 μm (B).

Figure 7 shows that the NT2 astrocytes express both GFAP (red), and Cx43 (green). Bar = 50 μm.

Figure 8 shows that dye coupling in NT2 astrocytes is readily demonstrated, using the scrape loading technique. NT2 astrocytes receive carboxyfluorescein from the brightly fluorescent donor cells labeled with both carboxyfluorescein and dextran tetramethylrhodamine (A). The scrape loaded donor cells are shown with arrows (B). Bar = 50 μm.

Figure 9 shows a preferred protocol for practicing the invention.

DETAILED DESCRIPTION OF THE INVENTION

The invention advantageously allows the differentiation of multipotential stem, such as the NT2/D1 progenitor cell line into replicating cells, such as astrocytes, which express connexin43 and are coupled via gap junctions. Thus, according to the invention, multipotential stem cells, such as human progenitor cells, are not merely committed neuronal progenitors but, similar to embryonal stem cells, they can give rise to different lineages.

The multipotential stem cells may comprise such cell lines as human progenitor cell line NT2/D1, mouse cell line P19, and other cell lines capable of multiple differentiation options. It was previously believed in the prior art that NT2/D1 cells were committed cells which differentiated only to neuronal cells. Advantageously, the process of the invention has illustrated that this cell line is indeed multipotential, and can differentiate to astrocytes for example.

Optionally, the culture of replicating cells according to the invention additionally comprises non-dividing cells. In particular embodiments, the non-dividing cells may be neurons. In one embodiment, the culture of replicating cells comprises at least 50% by number of replicating cells and less than 50% by number of non-dividing cells, more particularly, at least 55% by number of replicating cells.

In the embodiment of the invention where astrocytes are formed from multipotential stem cells, such as NT2/D1, various proportions of astrocytes may be formed in culture. Astrocytes are mitotic cells (i.e. they can divide), whereas neurons are post-mitotic (i.e. cannot divide). Therefore, the longer the cultures grow, the more astrocytes are developed and the ratio of astrocytes/neurons increases. For example, the percentage of astrocytes in the culture is relatively low at week 9, but the culture contains about 70% astrocytes at week 12, where weeks are considered from the beginning of the experiment, or at the start of step (a).

According to an embodiment of the invention, there is provided a process for *in vitro* preparation of astrocyte cells comprising (a) culturing multipotential stem cells in a medium containing retinoic acid; (b) culturing cells from step (a) in a medium containing from 0 to 0.7 μM cytosine arabinoside, from 0 to 7 μM fluorodeoxyuridine, and uridine for up to 1 week; (c) culturing cells resulting from step (b) in medium containing 0 to 7 μM fluorodeoxyuridine; and (d) then culturing cells resulting from step (c) in a medium containing uridine until astrocyte cells are produced.

In an optional embodiment, step (b) involves a medium containing cytosine arabinoside, fluorodeoxyuridine and uridine. According to this embodiment, an optional range for cytosine arabinoside is in an amount of 0.1 to 0.6 μM, preferably 0.3 to 0.5 μM, and especially 0.5 μM.

The step (b) incubation with an anti-mitotic agent, such as cytosine arabinoside, can occur 0 to 10 days, for example, from 1 to 7 days, preferably 7 days. In this embodiment, the cells are then cultured in a medium containing 0.1 to 0.7 μM, especially 0.4 to 0.6 μM, particularly 0.5 μM fluorodeoxyuridine for 4 days to 6 weeks, particularly 1 week to 4 weeks, especially up to 3 weeks. The cells are then cultured in a medium containing a non-toxic level of uridine, for example from 5 to 15 μM, especially about 10 μM uridine for at least about 2 weeks, particularly 4 weeks and often even longer, for example 6 to 20 weeks. The longer this incubation, the greater the ratio of replicative cells to non-replicative cells will become. The precise regime used is a matter of manipulation of concentration of compound and time of exposure. Dose can usually be regarded as a product of concentration of compound and time of exposure. Exposure to an anti-mitotic agent occurs at a level which is ___. For those periods of time which are very brief, and for the alternative wherein there is no incubation with an anti-mitotic agent, this is useful with the invention since very little killing of

replicative cells occurs, allowing such replicative cells as astrocytes and oligodendrocytes to develop from the culture. Because the anti-mitotic agent is used in the invention at a level which is less than that required to kill a replicating cell, the replicating cells evolving from the multipotential cells can survive in the culture. The prior art methodology uses levels of anti-mitotic agents high enough to kill replicating cells, and thus, have not resulted in cultures of replicating cells.

Once formed in culture, neurons tend to exist in the upper layers formed in culture, while the astrocytes tend toward the lower portions of culture. Thus, neurons can be removed from a culture of astrocytes formed with a relatively short uridine incubation period by removing neurons from on top of astrocytes in culture. In this way, a higher ratio of astrocytes can be obtained without increasing the uridine incubation period of step (c), according to the invention.

Although rodent brain cultures and neuronal and glial cell lines have provided us with important information about the structure and function of the mammalian CNS, studies using human neurons and astrocytes have been fairly limited. It is only recently that human NTera2 neurons (NT2N cells [1]) have been used as a model to study Alzheimer's disease (AD) [2,3], traumatic brain injuries [4,5], brain tumors [6] and other neurological problems [7]. The fact that NT2N cells survive, mature and integrate into the host CNS has made them a potentially strong candidate to treat human neurological diseases or traumas. In order to obtain NT2N cells, we [8,9] and others [7,10,11] have differentiated Ntera2/clone Dl (NT2/D1) cells, using retinoic acid (RA) and antiproliferative agents for 4 and 3 weeks, respectively. The total length of RA and post RA treatments to differentiate NT2/DI cells has been recently reduced to 3 weeks [12]; however, the purity of the neuronal cultures obtained is not well defined.

In the developing CNS, neurons and astrocytes are both formed from the proliferating neuroepithelial cells incorporated into the neural tube [13]. The prenatal neurogenesis process (4th to 20th week of pregnancy in humans) is followed by the formation of astrocytes (beginning at the 19th week), which serve as the largest syncytial compartment of the CNS [13,14]. Astrocytes are well equipped with various membrane receptors, which possibly respond to neuromodulators and neurotransmitters through signal transduction pathways [14]. The syncytial astrocytes are well connected to each other by gap junction channels that together provide for intercellular communication within the compartment [15]. Each gap

junction is made of two connexons (half channels) which are each a hexameric assembly of specific proteins called connexins [16]. This organization presumably allows astrocytes to provide a supportive role for neurons and other glia in the CNS [17]. The neuronal-glial partnership enhances neuronal survival and promotes axonal growth and synaptogenesis and possibly the efficacy of synaptic transmission [17-19]. The acquisition of mature neuronal phenotype in the CNS [20] as well as in NT2/Dl cultures [8] occurs concurrently with reduction of connexin43 (Cx43) and gap junctional communication, whereas astrocytes maintain Cx43 expression and intercellular coupling during the maturation process [21]. We hypothesized that, similar to the neuroepithelial cells *in vivo*, NT2/D1 cultures differentiate into neurons (NT2N cells) [8-11], followed by astrocytes (NT2A cells). We have also studied the functional aspect of NT2 astrocytes by examining the presence of gap junctions and intercellular coupling among these cells.

Materials and Methods. Cell culture: Cells were grown in Dulbecco's modified Eagle medium (DMEM), high glucose, glutamine (Gibco, BRL, Burlington, ON), 10% fetal bovine serum (FBS; Gibco, BRL) and 0.4gl/ml penicillin-streptomycin (Gibco, BRL), as described previously [10]. For differentiation studies, 2 X 10^6 cells were seeded per 75 cm^2 flask (Gibco, BRL) and treated with medium containing I0μM all-trans retinoic (RA, Sigma Chemical Co, Oakville, ON) for 4 weeks. Following RA treatment, cells were plated at a dilution of 1:6 in 60 mm tissue culture plates (Gibco, BRL) for 2 days, using RA free medium (replate 1).

In order to obtain pure neuronal cultures, the cells from replate 1 were seeded again (1 X 10^7 cells/ 60 mm plates) using the medium containing 1 μM cytosine arabinoside for 1 week, I0μM fluorodeoxyuridine and 10 μM uridine for 3 weeks (all from Sigma Chemical Co) (replate 2a).

In order to obtain astrocytes, the cells from replate 1 were seeded at a density of 1X10^7cells/60 mm plate in medium containing 0-0.5 μM cytosine arabinoside for 1 week, 5 μM fluorodeoxyuridine for 3 weeks and 10 μM uridine for > 4 weeks (replate 2b). A time period in excess of 12 or even 20 weeks may be used for the incubation with uridine, in order to increase the number of replicative cells with in the culture.

Particular examples of *in vitro* conditions including useful ranges of concentration and treatment times are listed in Table 1. Retinoic acid incubation concentrations and time

periods can be easily determined by one of skill in the art, given consideration to conventional methodologies for forming neuron cells.

Table 1

Exemplary *in vitro* Concentration and Treatment Duration

	Compound	Concentration	Treatment Duration
Example 1	Cytosine arabinoside Fluorodeoxyuridine Uridine	0.25 µmol/L 5 µmol/L 10 µmol/L	1 weeks 1 week 4 weeks
Example 2	Cytosine arabinoside Fluorodeoxyuridine Uridine	0.25 µmol/L 2.5 µmol/L 7 µmol/L	1 weeks 1 week 5 weeks
Example 3	Cytosine arabinoside Fluorodeoxyuridine Uridine	0.7 µmol/L 0 µmol/L 10 µmol/L	2 days 0 days 14 weeks

A preferred protocol is shown in Figure 9. The treatments outlined in the protocol merely show exemplary concentrations which can be used. The discontinuation of the anti-mitotic agent after about one week subsequent to the retinoic acid incubation may be followed by any combination of media incubation with uridine, and fluorodeoxyuridine need not be present in the incubation medium.

In order to determine the number of neurons and astrocytes, cells were counted in triplicate cultures after being labeled with Hoechst* dye (Sigma Chemical Co.) and MAP2 and GFAP antibodies.

Immunocytochemistry. The antibodies used in this study included a polyclonal connexin 43 antibody (Dr. Lau, University of Hawaii at Manoa), a monoclonal and a polyclonal glial fibrillary acidic protein (GFAP) antibody, monoclonal antibodies against microtubule associated protein 2 (MAP2a+2b), neurofilament (NF) 200 and vimentin (all from Sigma Chemical Co.), a fluorescein-conjugated goat antirabbit IgG (GAR-FITC) and a

*Trade-mark

Cy3-conjugated goat anti-mouse IgG (GAM-Cy3; both from Jackson Immunoresearch Laboratories, Inc., West Grove, PA).

Cells were plated in 60mm tissue culture dishes and fixed with 70% ethanol containing 0.15 M NaCl for 10min[8-10]. Non-specific binding sites were blocked with 10% normal goat serum (NGS; Dimension Labs, Mississauga, ON) for 30min. Cells were washed with PBS (pH 7.4) and incubated with primary antibody for 1 h. After three washes (10 min each) cells were incubated with secondary antibody (GAR-FITC or GAM-Cy3) for 1 h, washed with PBS (three times, 10min each) and covered with Vectashield* mounting medium (Vector Laboratories, Burlingame, CA) and coverslip. Images were captured using a Zeiss* Axioskop* microscope equipped with filter sets for phase contrast, fluorescein and rhodamine, and Northern Eclipse* imaging software (ImageExperts Inc., Mississauga. ON).

Dye Coupling (Scrape Loading). Dye transfer in the undifferentiated and differentiated cultures was tested at confluency in 60mm tissue culture plates. The medium was aspirated from the plate and a small square border (about 2 X 2 cm^2) was scraped through the middle of the plate, using a glass Pasteur pipet. A volume of 50µl dye mixture (0.1% 5(6)-carboxyfluorescein (mol. wt 376.3, Sigma Chemical Co) and 0.1% dextran tetramethylrhodamine (mol. wt 3000, Molecular Probes, Eugene, OR) in PBS, pH 7.4) was added inside the scraped border. In order to label donor cells, a razor blade was run across the square several times to allow penetration of dextran tetramethylrhodamine as well as carboxyfluorescein. After 2min incubation, cells were washed (several times) and covered with PBS. Images were captured immediately, as outlined above.

Results. NT2/D1 cells were negative for MAP2 (Fig. 1A) and NF (Fig. 1B), whereas they expressed an abundant amount of Cx43 (Fig. 1C) before RA induction, as expected [8,9]. These cells differentiated into a pure (about 97 ± 2%, n = 20) population of MAP2 and NF positive neurons (NT2N cells) after treatment with RA and antiproliferative agents (Fig. 1D, E, respectively) and lost the Cx43 expression (Fig. IF). NT2N cultures were negative for GFAP, as shown previously [8,9]. Alternatively, NT2/D1 cells differentiated into both MAP2 positive neurons (30 ± 3%, n = 20) and GFAP positive astrocytes (NT2A cells; Fig. IG), using a lower concentration of the antiproliferative agents over a longer period (> 4 weeks). The latter developed into a large number (62 ± 5%, n = 20) of mature Cx43 positive astrocytes after 8 weeks of antiproliferative treatment (Fig. 1H). The NT2A cultures were

negative for vimentin (Fig. 1I). The post-mitotic NT2 neurons survived longer, in the presence of NT2 astrocytes (about 6 months) than did the pure NT2 neuronal cultures (2-3 months). NT2 astrocytes labeled with 5(6)-carboxyfluorescein (mol. wt 376.3) and dextran tetramethylrhodamine (mol. wt 3000) readily transferred 5(6)-carboxyfluorescein into the adjacent cells (Fig. 2). NT2 neurons did not show coupling (data not shown), as reported previously [8].

Discussion. In this study, we have surprisingly shown that human teratocarcinoma NT2/DI cells have the capacity to differentiate into both neurons (NT2N cells) and astrocytes (NT2A cells) by modifying the originally published protocol [10]. In addition, we have shown that NT2 astrocytes express Cx43 and communicate through gap junctions. Cytosine arabinoside and fluorodeoxyuridine are DNA synthesis inhibitors and increase the proportion of post-mitotic neurons by decreasing the number of non-neuronal cells in the culture [10,11,22,23]. A reduction in concentration of these compounds plus the use of a later developmental window lead to the formation of astrocytes in these cultures.

Other studies have shown that primary neurons grown in co-culture with astrocytes form functional synapses with enhanced synaptic frequency, compared to neurons grown in the absence of glia [14,24]. In order to study human neurons, the lack of human astrocytes has been addressed by using astrocytes from animal sources [7]. Most recently, rat astrocytes have been used as a supportive layer to promote the maturation, survival and synaptogenesis of NT2 neurons [19]. In the absence of astrocytes, NT2 neurons rarely form synapses and they have weak action potentials [19]. It seems that the presence of a direct contact between NT2 neurons and astrocytes, and not merely treatment with astrocytic conditioned medium, is necessary for optimal growth and synaptogenesis of NT2 neurons [19].

In our experiments, pure NT2 neuronal cultures survived for about 2-3 months, whereas those in contact with NT2 astrocytes maintained a healthy status for more than 6 months. It is possible that astrocytes regulate the delivery of nutrients to the adjacent neurons and/or the removal of waste neuronal products from the extracellular regions by way of gap junctions [14,25]. More specifically, supplying neurons with glucose and clearing excess extracellular K+ and H+ ions are potentially the most prominent roles of astrocytic gap junctions [14]. NT2N-NT2A cultures are a useful model to study the interaction between human neurons and

glia, providing insight into the dual trophic and protective function of astrocytes in the human CNS.

In the embodiment of the invention wherein the multipotential stem cells are progenitor cells and the replicating cells produced are astrocytes or oligodendrocytes, the invention is advantageous and useful in treatment of various forms of neurological deficiency. Astrocyte cells, and mixed neuron and astrocyte cultures prepared according to the invention can be used in the treatment of conditions or diseases of the brain, spinal cord or nerves. Examples of such conditions or diseases include stroke, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, or for relief of trauma or injury to the brain, spinal cord or nerves. Cell cultures can be utilized as a therapy for such conditions, for example through direct delivery or implantation to the brain. Any condition or disease in which neurons and astrocytes would provide beneficial results could be treated with cells formed according to the invention. A cell therapy regime, which is useful in repair or enhancement of biological function for the damaged brain, spinal cord or nerves. For a review of cell therapy, see Gage F.H. (1998) Nature, Vol. 392, Suppl., p. 18-24. Compositions for use in cell therapy include a culture of cells according to the invention in combination with a pharmaceutically acceptable carrier.

Further, cells and cell cultures prepared according to the invention can be used as an *in vitro* model of the brain for such applications as drug efficacy testing, for high-throughput screening methods in candidate drug identification, particularly for identification of drugs which effect brain function. Any application in which neuron cells are used would benefit from the cell culture according to the invention, since astrocytes provide a neuroprotective function, and neurons survive longer in the presence of astrocytes.

References

1. Andrews PW. Damianov 1, Simon D et al Lab Invest 50. 147-162 (1984).
2. Hung AY, Koo EH, Haass C and Selkoe DJ. Proc Natl Acad Sci USA 89, 9439-9443 (1992).
3. Wertkin AM. Turner RS, Pleasure SJ et al. Proc Natl Acad Sci USA 90, 9513-9517 (1993).
4. La Placa MC and Thibault LE. Ann Biomed Eng 25, 665-677 (1997).

5. La Placa MC and Thibault LE. J Neurosci Res 62. 220-229 (1998).
6. Kesari S. Randazzo BP, Valyi-Nagy T et al. Lab Invest 73, 636~48 (1995).
7. Trojanowski JO, Kleppner SR. Hartley RS et al. Exp Neurol 144, 92~97 (1997).
8. Bani-Yaghoub M, Bechberger JF and Naus CCG. J Neurosci Res 49, 19-31 (1997).
9. Bani-Yaghoub M, Bechberger JF, Urbderhill MT and Naus CCG. Exp Neurol 156, 16-32 (1999).
10. Pleasure SJ, Page C and Lee VMY, J Neurosci 12, 1802-1815 (1992).
11. Pleasure SJ and Lee VMY. J Neurosci Res 35. 585-602 (1993).
12. Cheung WM. Fu WY. Hui WS and lp NY. Biotechniques 26, 946 (1999).
13. Kiernan JA. The Human Nervous System. Philadelphia: Lippincott-Raven, 1998: p.4.
14. Froes MM and Campos De Carvalho AC. Glia 24, 97-107 (1998).,
15. Jeffreys JG. Physiol Rev 75, 689-723 (1995).
16. Bruzzone R, White TW and Paul DL. Eur J Biochem 238, 1-27 (1996).
17. Smith SJ. Curr Biol 26, R158 -60 (1998).
18. Barres BA. Neuron-glial interactions. In: Cowan WM, Jessel TM and Zipursky SL, eds. Molecular and Cellular Approaches to Neural Development New York: Oxford University Press. 1997: 64-107.
19. Hartley RS, Margulis M, Fishman PS et al J Comp Neurol 407, 1-10 (1999).
20. Kandler K and Katz LC. Curr Opin Neurobiol 5 98-105 (1995).
21. Giaume C and Venance L. Perspect Dev Neurobiol 2, 335-45 (1995),
22. Ivilijn MH, Vaysse PJ, Zukin RS and Kessler JA. Proc Nad Acad Sci USA 85, 6551-5 (1988).
23. Abraham I, Sampson KE, Powers EA et al. J Neurosci Res 28, 29-39 (1991).
24. Pfrieger FW and Barres BA. Science 277, 1684-7 (1997).
25. Giaume C and McCarthy KD. Trends Neurosci 19, 319-25 (1996).

The above-described embodiments of the invention are intended to be examples of the present invention. Alterations, modifications and variations may be effected the particular embodiments by those of skill in the art, without departing from the scope of the invention which is defined solely by the claims appended hereto.

CLAIMS:

1. A culture of replicating cells produced by (a) culturing multipotential stem cells in a medium containing retinoic acid, (b) incubating the cells from step (a) in a medium containing an anti-mitotic agent at an amount less than that required to kill a replicating cell, and (c) subsequently incubating cells in a medium containing uridine until replicating cells are produced.

2. The culture of cells according to claim 1, wherein said multipotential stem cells are progenitor cells, and said replicating cells are selected from the group consisting of astrocytes and oligodendrocytes.

3. The culture of cells according to claim 1 or 2, wherein said multipotential stem cells are NT2/D1 cells or P19 cells.

4. A culture of replicating cells produced by (a) culturing multipotential stem cells in a medium containing retinoic acid, (b) incubating the cells from step (a) in a medium containing cytosine arabinoside at an amount less than that required to kill a replicating cell, and (c) subsequently incubating cells in a medium containing uridine until replicating cells are produced.

5. A culture of cells according to claim 4, wherein said replicating cells are astrocytes.

6. A culture of cells according to claim 4 or 5, additionally comprising neuron cells.

7. A culture of cells according to claim 4, 5 or 6, comprising at least 50% by number of astrocytes and less than 50% by number of neuron cells.

8. A culture of cells according to claim 7 comprising at least 55% by number of astrocytes.

9. A culture of cells according to claim 8 comprising 62% ± 5% by number of astrocytes and 30% ± 3% by number of neuron cells.

10. A process for producing replicating cells from multipotential stem cells comprising the steps of: (a) culturing multipotential stem cells in a medium containing retinoic acid, (b) incubating the cells from step (a) in a medium containing an anti-mitotic agent at an amount less than that required to kill a replicating cell, and (c) subsequently incubating cells in a medium containing uridine until replicating cells are produced.

11. The process according to claim 10, wherein said anti-mitotic agent is cytosine arabinoside.

12. A process for *in vitro* preparation of astrocyte cells comprising (a) culturing multipotential stem cells in a medium containing retinoic acid, (b) culturing cells in a medium containing less than 0.7 μM cytosine arabinoside and containing less than 7 μM fluorodeoxyuridine; and (c) then culturing cells resulting from step (b) in a medium containing uridine until astrocyte cells are produced.

13. The process according to claim 12, wherein in step (b) said cells are cultured in a medium containing 0.2 to 0.6 μM cytosine arabinoside and 2 to 6 μM fluorodeoxyuridine, and in step (c) said cells are cultured in a medium containing 5 to 15 μM uridine.

14. The process according to claim 13 wherein in step (b) said cells are cultured in a medium containing 0.5 μM cytosine arabinoside, and 5 μM fluorodeoxyuridine and in step (c) said cells are cultured in a medium containing 10 μM uridine.

15. The process according to any one of claims 10 to 14, wherein said multipotential stem cells are NT2/D1 or P19 cells.

16. Use of the culture of cells according to any one of claims 1 to 9 for prevention, treatment, or mitigation of a condition or disease of the brain, spinal cord or nerves.

17. The use according to claim 16, wherein said condition or disease of the brain, spinal cord or nerves is selected from the group consisting of stroke, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, trauma, and injury.

18. The use according to claim 16 or 17, wherein said culture of cells is delivered by cell therapy.

19. A composition for use in cell therapy comprising a culture of cells according to any one of claims 1 to 9 in combination with a pharmaceutically acceptable medium.

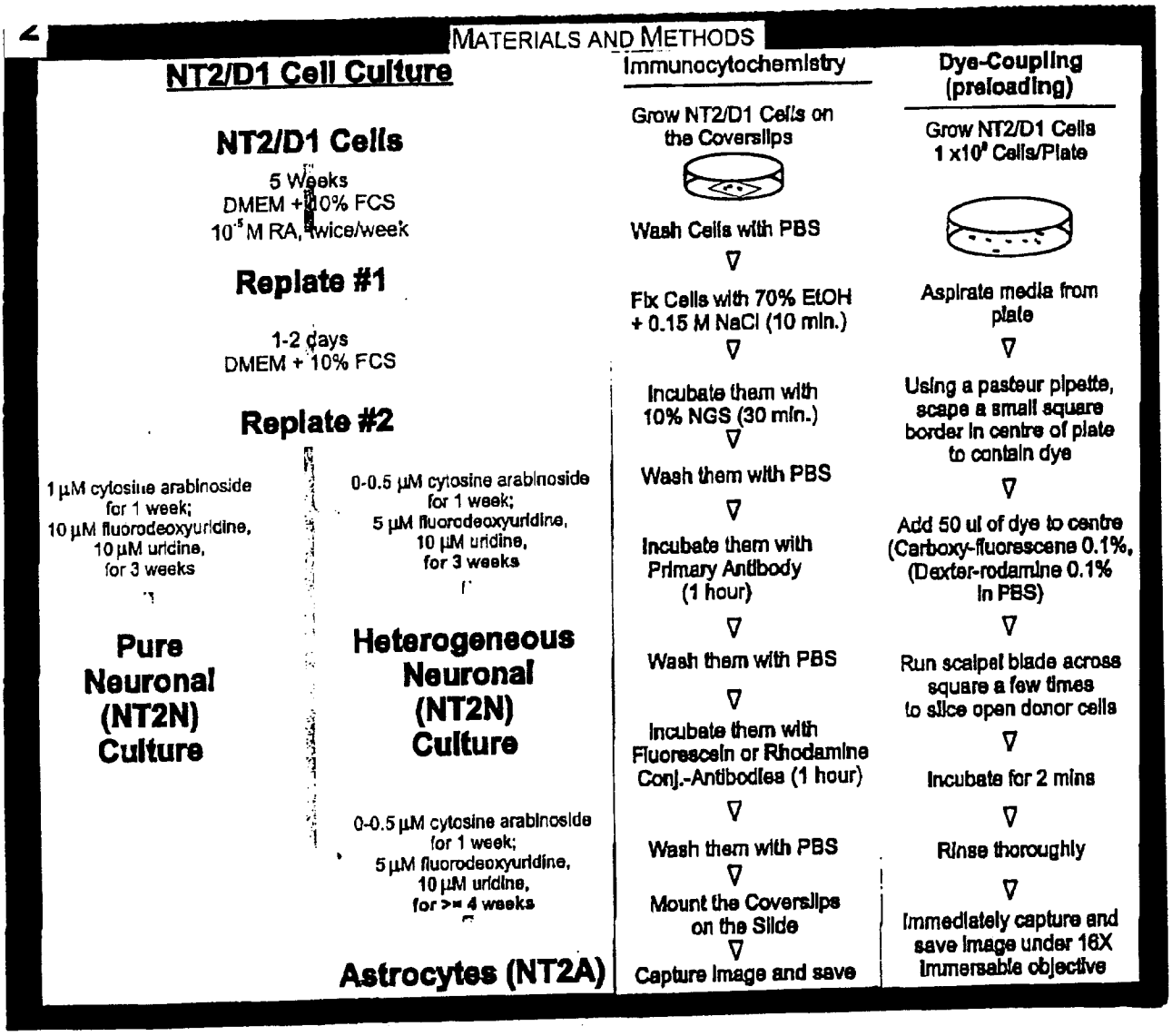
MATERIALS AND METHODS
NT2/D1 Cell Culture
NT2/D1 Cells
5 Weeks
DMEM + 10% FCS
10⁻⁵ M RA, twice/week
Replate #1
1-2 days
DMEM + 10% FCS
Replate #2
1 µM cytosine arabinoside for 1 week; 10 µM fluorodeoxyuridine, 10 µM uridine, for 3 weeks
Pure Neuronal (NT2N) Culture
0-0.5 µM cytosine arabinoside for 1 week; 5 µM fluorodeoxyuridine, 10 µM uridine, for 3 weeks
Heterogeneous Neuronal (NT2N) Culture
0-0.5 µM cytosine arabinoside for 1 week; 5 µM fluorodeoxyuridine, 10 µM uridine, for >= 4 weeks
Astrocytes (NT2A)
Immunocytochemistry
Grow NT2/D1 Cells on the Coverslips
Wash Cells with PBS
Fix Cells with 70% EtOH + 0.15 M NaCl (10 min.)
Incubate them with 10% NGS (30 min.)
Wash them with PBS
Incubate them with Primary Antibody (1 hour)
Wash them with PBS
Incubate them with Fluorescein or Rhodamine Conj.-Antibodies (1 hour)
Wash them with PBS
Mount the Coverslips on the Slide
Capture Image and save
Dye-Coupling (preloading)
Grow NT2/D1 Cells 1 x10⁶ Cells/Plate
Aspirate media from plate
Using a pasteur pipette, scape a small square border in centre of plate to contain dye
Add 50 ul of dye to centre (Carboxy-fluorescene 0.1%, (Dexter-rodamine 0.1% in PBS)
Run scalpel blade across square a few times to slice open donor cells
Incubate for 2 mins
Rinse thoroughly
Immediately capture and save image under 16X Immersable objective

FIG. 9

UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

232 2554

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA

PRÉPARATION DES DOSSIERS)

MATERIALS AND METHODS

NT2/D1 Cell Culture

NT2/D1 Cells

5 Weeks
DMEM + 10% FCS
10^{-5} M RA, twice/week

Replate #1

1-2 days
DMEM + 10% FCS

Replate #2

1 µM cytosine arabinoside
for 1 week;
10 µM fluorodeoxyuridine,
10 µM uridine,
for 3 weeks

Pure Neuronal (NT2N) Culture

0-0.5 µM cytosine arabinoside
for 1 week;
5 µM fluorodeoxyuridine,
10 µM uridine,
for 3 weeks

Heterogeneous Neuronal (NT2N) Culture

0-0.5 µM cytosine arabinoside
for 1 week;
5 µM fluorodeoxyuridine,
10 µM uridine,
for >= 4 weeks

Astrocytes (NT2A)

Immunocytochemistry

Grow NT2/D1 Cells on the Coverslips

Wash Cells with PBS

∇

Fix Cells with 70% EtOH + 0.15 M NaCl (10 min.)

∇

Incubate them with 10% NGS (30 min.)

∇

Wash them with PBS

∇

Incubate them with Primary Antibody (1 hour)

∇

Wash them with PBS

∇

Incubate them with Fluorescein or Rhodamine Conj.-Antibodies (1 hour)

∇

Wash them with PBS

∇

Mount the Coverslips on the Slide

∇

Capture Image and save

Dye-Coupling (preloading)

Grow NT2/D1 Cells
1 x10^6 Cells/Plate

Aspirate media from plate

∇

Using a pasteur pipette, scape a small square border in centre of plate to contain dye

∇

Add 50 ul of dye to centre (Carboxy-fluorescene 0.1%, (Dextar-rodamine 0.1% in PBS)

∇

Run scalpel blade across square a few times to slice open donor cells

∇

Incubate for 2 mins

∇

Rinse thoroughly

∇

Immediately capture and save image under 16X Immersable objective